Chemical Mutagenesis of *Saccharomyces cerevisiae* for Enhancing Bioethanol Production with Fermentation at Very High Sugar Concentration

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Saccharomyces cerevisiae is one of the most promising unicellular fungi on account of its vital applications in biotechnology as well as bioethanol production. Improvement of ethanol production *via* very high-gravity (VHG) fermentation (fermentation at high sugar levels) was successfully developed using the ethidium bromide (EtB) mutagenesis of *S. cerevisiae*. This study found two developed mutants of *S. cerevisiae* (EtB20a and EtB20b) with varied capacity for ethanol production using EtB, depending on random amplified polymorphic DNA analysis. Mutant EtB20b showed improved ethanol yield (19.5%) compared with the wild-type (18.0%), while the other mutant EtB20a exhibited retarded ethanol production (9.1%). Optimization of ethanol production by mutant EtB20b was performed under other conditions including temperature, pH, inoculum size, and incubation period. The highest production capacity of the yeasts was 20.8, 19.9, 19.5, and 19.5% at an optimum temperature of 30 °C, pH 6.0, incubation period of 72 h, and 1 mL of yeast suspension (optical density at 600 nm) with glucose utilization of 42.6, 40.7, 39.8, and 39.9%, respectively.

Keywords: Bioethanol; Very high gravity fermentation; Mutagenesis; Saccharomyces cerevisiae

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INTRODUCTION

Each year all kinds of industries increase globally; therefore, energy research has also dramatically increased. Several environmental problems and global climate change rapidly appeared as a result of fossil fuels' and their derivatives' consumption. In recent years, bioenergy as well as bioethanol demand has become greater than before as a source of eco-friendly and safe alternative energy. Production of bioethanol can contribute to the solution of these problems due to its nature as a clean, renewable, and carbon-neutral fuel (Farrell *et al.* 2006; Hill *et al.* 2006). The industrial development and continuous rise of crude oil cost has improved the competitiveness of bioethanol against fossil fuels. Over the past years, discussion and focus on the green impacts of bioethanol and other biofuels have appeared (Anex and Lifset 2009).

Yeasts, particularly the *Saccharomyces* genus, are usually selected for the production of alcoholic products due to various reasons, such as a high ethanol yield more than 5.0 g/L/h, a tolerance to high ethanol concentration, growth under stress physical conditions, such as pH and temperature, growth in uncomplicated, cheap, and undiluted

media, as well as its ability to grow in the presence of inhibitors such as furfural and toxins (Afifi *et al.* 2011; Zarif *et al.* 2011; El-Taher *et al.* 2012; López-Malo *et al.* 2013; Raffaela and Laura 2017). Many authors (Dhabekar and Chandak 2010; Abdel Ghany *et al.* 2014; Nuanpeng *et al.* 2018) reported that *S. cerevisiae* is used as a widespread producer for ethanol. The *S. cerevisiae* species is usually considered the preferable yeast for wine and cider fermentations. However, there is another species of the *Saccharomyces* genus, namely *S. bayanus*, that is utilized for the manufacturing of cider, wine, and sparkling wines, and it can also be applied in the industrial production of bioethanol (Publicover *et al.* 2010). Scientific papers have researched yeast strains for use in ethanol production (Farman *et al.* 2010; Mussato *et al.* 2012). For example, *Pichia stipitis* and *Kluyveromyces fagilis* were reported as excellent ethanol producers from various types of sugars. Generally, yeasts during the fermentation process suffer from different stresses such as hyperosmolarity and inhibition due to elevated ethanol levels or rising temperature (Gibson *et al.* 2007; Caspeta *et al.* 2015). Stanley *et al*. (2010a) reported that yeasts, when grown in substrates with high concentrations of sugar, are suddenly exposed to osmotic stress that effects yeast viability and ethanol yield.

The term of very high gravity (VHG) fermentation is applied to media containing more than 25% sugar, and for enhancing ethanol yield more than 15% (v/v) (Puligundla *et al.* 2011). In addition, it increases productivity, minimizes energy, reduces water consumption, and shortens processing period of fermentation (Bayrock and Ingledew 2001; Yang *et al.* 2019). Yeast cells in VHG fermentation media suffer from two problems, which are osmotic and ethanol stress, due to high sugar level at an early stage and high ethanol yield at the end stage of fermentation, respectively (Rautio *et al.* 2007). Researching for stress tolerant yeast strains are economic characteristics for ethanol production.

Engineering yeast strains plays an important role for a successful VHG fermentation through improved tolerance to high ethanol and sugar levels (Hou 2010; Zarif *et al.* 2011; Wei *et al.* 2013; Pattanakittivorakul *et al.* 2019). Numerous authors have reported that mutants not only tolerate these stresses but also exhibit resistance to high temperature, oxidative stresses, and other inhibitors (Zarif *et al.* 2011; Kumari and Pramanik 2012; Zhang *et al.* 2015; Pattanakittivorakul *et al.* 2019). This study aimed to investigate the mutagenesis of *S. cerevisiae* by ethidium bromide for enhancing bioethanol production in VHG fermentation and the optimal production conditions.

EXPERIMENTAL

Materials

Yeast used and maintenance of culture

Saccharomyces cerevisiae was obtained from the Egyptian Sugar and Integrated Industries Company, Cairo, Egypt. Yeast was activated and maintained by subculturing on yeast extract peptone dextrose (YEPD) agar medium, incubating for 48 h at 28 °C, and, thereafter, storing in a refrigerator until future use.

VHG ethanol fermentation medium

The VHG fermentation medium contained (g/L^{-1}): $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 1.0; $CaCl_2{\cdot}2H_2O$, 0.1; NaCl, 0.1; yeast extract 3.0 and glucose 300 were used for ethanol fermentation. For studying the high osmotic stress, 400 and 500 g/L^{-1}

glucose were added to the fermentation medium. These media were inoculated by a fresh culture of *S. cerevisiae* and then incubated at 28 °C for 72 h.

Mutation induction by EtB

Suspension of wild-type *S. cerevisiae* was diluted by using distilled water with a ratio of 1:10 and transferred to sterile test tubes. Ethidium bromide (EtB) was added at different concentrations of 5, 10, 15, 20, and 25 µg/mL, and the culture was incubated at room temperature for 30 min. Then, 0.1 M phosphate buffer (pH 7.1) was added to each test tube containing the dilution, which was required to stop the mutagenesis. The dilutions were spread on the YEPD plates. Distilled water without any EtB additive was used in the control cultures.

Methods

Assessment of ethanol production and reducing sugars

After inoculation and incubation of the wild-type and mutant yeast in VGH fermentation medium, 1 mL of fermented wash was added to 30 mL of distilled H_2O in a 500 mL Pyrex distillation flask. The collected distillate was added to 25 mL of the reagent potassium dichromate (Carl-Roth, Karlsruhe, Germany) (33.768 g of $K_2Cr_2O_7$ dissolved in 400 mL of distilled water with 325 mL of H_2SO_4, and the volume completed to 1 L). Then, 20 mL of the sample in the flasks was kept in a water bath maintained at 62.5 ºC for 20 min, then cooled to 25 °C, and diluted with distilled water up to 50 mL. Five mL of diluted sample was added to its equal volume of distilled water followed by measuring the optical density at 600 nm using a spectrophotometer (Model Jenway 6300; Cole-Parmer Ltd., Eaton Socon, England). Under a similar set of conditions, a standard curve was prepared using different concentrations of ethanol (Fig. 1) (Caputi, Jr. *et al.* 1968). The reducing sugar was estimated using dinitrosalycylic acid (DNS) (Carl-Roth, Karlsruhe, Germany) according to Miller (1959). Reducing sugar concentration was estimated from the standard curve of glucose (prepared by using 100 to 1000 mg concentration prepared in distilled water) (Fig. 2).

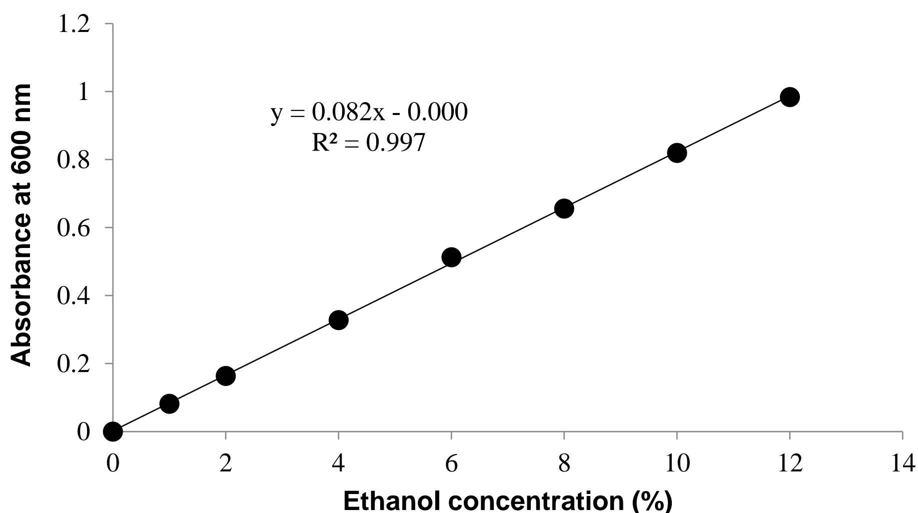


Fig. 1. Ethanol standard curve

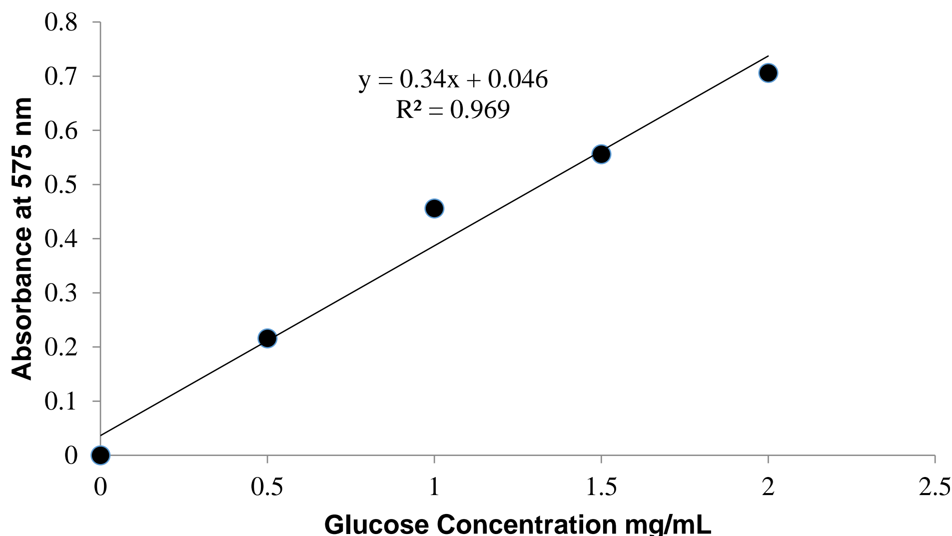


Fig. 2. Standard curve for estimation of reducing sugars

Analysis of EtB mutants using random amplified polymorphic DNA (RAPD) DNA extraction

For DNA extraction, *S. cerevisiae* after cultivation overnight in YEPD broth, 5 mL aliquots of culture were spun into pellet cells. The pellet was resuspended in 500 µL of sterile distilled water and transferred to a 1.5-mL tube followed by vortexing. Each pellet was spun again and the supernatant removed. The pellet was resuspended in 200 µL of lysis buffer (2% Triton X-100 and 1% sodium dodecyl sulfate), 200 µL of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and 300 mg acid-washed glass beads. The mixture was vortexed for 8 min, followed by centrifugation at 10000 rpm for 5 min in a microcentrifuge (Minispin; Eppendorf AG, Hamburg, Germany). The aqueous (top) layer was carefully transferred to another tube containing 1 mL 100% ethanol, followed by centrifugation at 10000 rpm for 2 min and the supernatant was removed. For the pellet, 10 µL ammonium acetate (4 M) and 1 mL ethanol (100%) were added, and then the pellet was spun for 2 min. Lastly, the obtained pellet was dried and resuspended in 50 µL Tris-EDTA buffer (Hoffman and Winston 1987).

DNA amplification

Reactions of PCR (volume 25 µL) with random primers contained 25 to 50 nanogram (ng) of DNA (1 µL of diluted DNA), 1.5 µL of 10× reaction buffer, 200 µM deoxynucleotide triphosphates (dNTPs), 0.6 units Taq polymerase, and 0.8 µM primer (Operon Technologies Inc., Alamdea, CA, USA). The mixture was overlain with 40 µL of sterile light mineral oil and placed on a thermocycler (Thermal cycler 2400; PerkinElmer, Inc., Waltham, MA, USA). Cycling circumstances incorporated an initial 4 min melt at 93 °C followed by 44 cycles for 1 min at 92 °C, for 1 min at 37 °C, and for 2 min at 72 °C. The last cycle was for 1 min at 92 °C, for 1 min at 37 °C, and for 8 min at 72 °C. Products of PCR were separated in 1.8% agarose gels electrophoresis (Msminiduo; Sigma-Aldrich, St. Louis, MO, USA) with 100 Kb DNA ladder (Invitrogen; Thermo Fischer Scientific, Waltham, MA, USA) as a DNA marker. The primers were screened and all tested primers that produced strong, reproducible PCR products (bands) were selected for further study.

The reproducibility of the RAPD markers was tested by performing PCR reactions with different concentrations (20 to 200 ng) of DNA template, with at minimum three independent DNA extractions from the identical sample. The primers used in this study are listed in Table 1.

Table 1. Primers Used

Primer Name	Primer Sequences
OPB-09	TGGGGGACTC
OPD-02	GGACCCAACC
OPE-04	GTGACATGCC
OPE-05	TCAGGGAGGT
OPL-12	GGGCGGTACT

Optimization of ethanol production and sugar utilization by mutant EtB20b

For pH optimization, VGH fermentation medium was prepared with different pH ranging from 3 to 9 in a volume of 100 mL media in a 250-mL conical flask. A total of 1 mL of 24-h-old mutant EtB20b (OD at 600 nm) was inoculated and then incubated for 72 h at 30 °C. For temperature optimization, the same conditions of the above medium were utilized except the inoculated media was adjusted to pH 6 and incubated at different temperatures ranging from 20 °C to 40 °C. For the incubation period optimization, the inoculated medium was adjusted to pH 6 and incubated for different times ranging from 12 to 84 h. To optimize the required size of the inoculum, the prepared medium was inoculated with different volumes of inoculums that ranged from 0.5 to 3 mL of 24-h-old (0.01 at 600 nm) mutant EtB20b. At the end of the incubation period for each parameter, the ethanol production and sugar utilization were estimated using Caputi, Jr. *et al.* (1968) and Miller (1959), respectively.

Statistical analysis

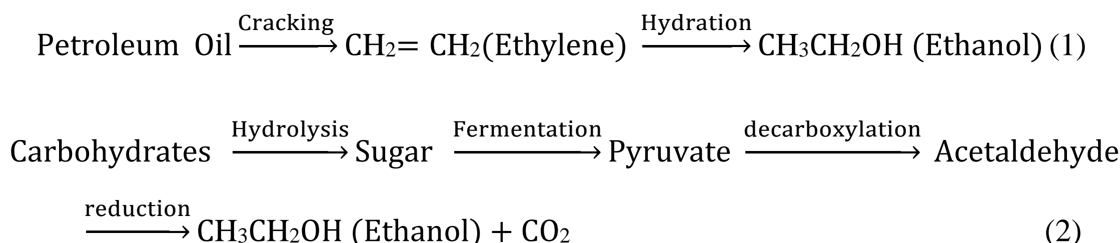
Statistical analysis of the obtained data was preformed using SPSS Statistics for Windows (IBM Corp., Version 20.0. Armonk, NY, USA) according to the procedure of Sendecor and Cochran (1981) and the means were compared using a multiple range test of Duncan (1988).

RESULTS AND DISCUSSION

Mutation Induction by EtB

Two main methods were used for ethanol production. The process was carried out either chemically (Eq. 1) through hydration of ethylene through the petroleum cracking or microbiologically (Eq. 2), where the main bioreaction of microbial fermentation including the converting hexose into two molecules of ethanol and carbon dioxide through formation of pyruvate which then decarboxylates by pyruvate decarboxylase into acetaldehyde, which further reduced by alcohol deydrogenase to ethanol. However, microbiologically method as well as using of wild or mutant yeasts for ethanol production are preferably and characterized by low formation of byproducts.

The production of ethanol by yeasts is characterized by high selectivity and low formation of byproducts, as shown in Eqs. 1 and 2.



$$\text{Petroleum Oil} \xrightarrow{\text{Cracking}} CH_2{=}\ CH_2(\text{Ethylene}) \xrightarrow{\text{Hydration}} CH_3CH_2OH\ (\text{Ethanol}) \quad (1)$$

$$\text{Carbohydrates} \xrightarrow{\text{Hydrolysis}} \text{Sugar} \xrightarrow{\text{Fermentation}} \text{Pyruvate} \xrightarrow{\text{decarboxylation}} \text{Acetaldehyde}$$

$$\xrightarrow{\text{reduction}} CH_3CH_2OH\ (\text{Ethanol}) + CO_2 \quad (2)$$

The development of new strains of *Saccharomyces cerevisiae* tolerance to stresses as well as high ethanol productivity is favored as a sustainable solution to biofuel production. In the current study, 24-h-old *S. cerevisiae* culture was exposed to different concentrations of EtB including 5, 10, 15, 20, and 25 µg/mL. Subcultured *S. cerevisiae* on YEPD agar indicated that the death rate increased with increasing EtB concentration. The colony count was 99, 64, 48, 25, 8, and 0 at 0, 5, 10, 15, 20, and 25µg/mL EtB, respectively. The *S. cerevisiae* colony count was recorded and a dose response curve was generated (Fig. 3). The safety data concerning EtBr appears to be contradictory (Sayas *et al.* 2015). Some authors have shown that the mutation of various genes occurs under EtBr exposure (Pinto *et al.* 1975; Pfeiffer *et al.* 2010; Stachowiak 2013; Sayas *et al.* 2015). In the present study, the mutants were selected from those that survived over 20 µg/mL EtB and at the same time they were subcultured on 10% ethanol YEPD medium to test their tolerance to high ethanol concentration. Two of the cultures developed mutants of *S. cerevisiae* (EtB20a and EtB20b). The potential mutants were grown numerous times and streaked until it became stable.

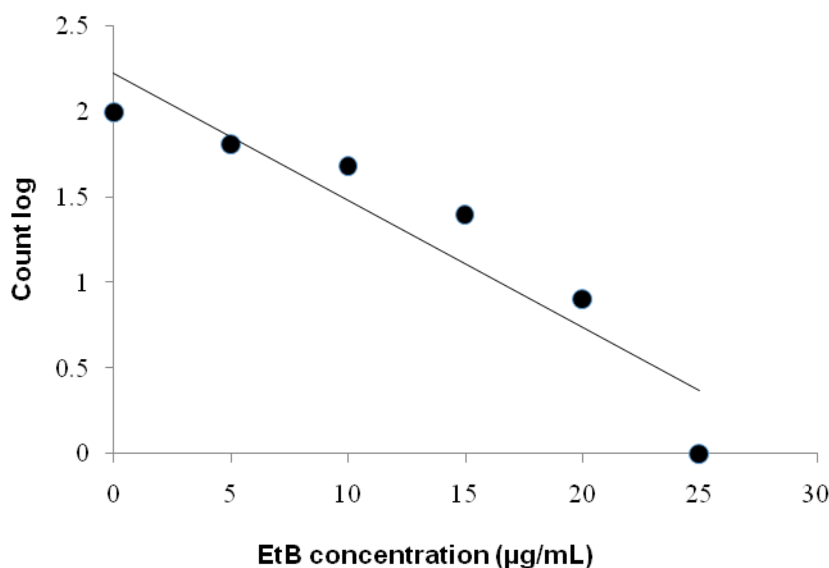


Fig. 3. *S. cerevisiae* colonies count at different EtB concentrations

Ethidium bromide selected mutants (EtB20a and EtB20b) and wild-type *S. cerevisiae* underwent RAPD analysis using random primers. From PCR product underwent agarose gel electrophoresis, the gel was documented and analyzed (Fig. 4 and Table 2). The genomic DNA of the wild-type and mutant strains (EtB20a and EtB20b) was analyzed by the RAPD-PCR technique. This test was applied to confirm that the developed mutant strains were genetically different from those of the wild-type. Five primers including, OPB-09, OPD-02, OPE-04, OPE-05, and OPE-05, amplified polymorphic DNA fragments in

the mutant strains. According to the OPB-09 primer, data in Table 2 show that bands with 3411.279, 2251.026, 1646.509, 958.009, and 278.438 base pair molecular weights were common bands in the wild-type, mutant EtB20a, and mutant EtB20b. Two bands with 1040.68 and 550.9 base pair molecular weights were detected only in the mutants unlike bands with 1115.89 and 469.506 base pair molecular weight. The EtB20b mutant was characterized by the presence of a band with 330.852 base pair molecular weight.

Table 2. Plus/Minus Data for Primers Gel Image

Primer Name	MW	Wild-type	EtB20a	EtB20b	Polymorphism
OPB-09 primer	3411.279	+	+	+	Polymorphic
	2251.026	+	+	+	Polymorphic
	1646.509	+	+	+	Polymorphic
	1115.890	+	-	-	Monomorphic
	1040.680	-	+	+	Polymorphic
	958.009	+	+	+	Polymorphic
	550.900	-	+	+	Polymorphic
	469.506	+	-	-	Monomorphic
	330.852	-	-	+	Monomorphic
	278.438	+	+	+	Polymorphic
OPD-02 primer	2251.026	+	+	+	Polymorphic
	1646.509	+	+	+	Polymorphic
	1115.890	+	+	+	Polymorphic
	958.009	+	+	+	Polymorphic
	819.334	+	-	-	Monomorphic
	727.974	+	+	+	Polymorphic
	649.272	+	+	+	Polymorphic
	550.900	-	+	+	Polymorphic
	469.506	+	+	+	Polymorphic
	278.438	+	+	+	Polymorphic
OPE-04 primer	2251.026	+	+	+	Polymorphic
	1646.509	+	+	+	Polymorphic
	1115.890	+	+	+	Polymorphic
	958.009	+	+	+	Polymorphic
	828.762	+	-	-	Monomorphic
	727.974	+	+	+	Polymorphic
	622.609	-	+	+	Polymorphic
	278.438	+	-	+	Polymorphic
OPE-5 primer	958.009	+	+	+	Polymorphic
	469.506	-	+	-	Monomorphic
	330.852	-	+	-	Monomorphic
	293.318	+	+	+	Polymorphic
	278.438	+	+	+	Polymorphic
OPL-12 primer	1040.479	+	+	+	Polymorphic
	958.009	+	+	+	Polymorphic
	789.487	+	+	+	Polymorphic
	499.828	+	+	+	Polymorphic

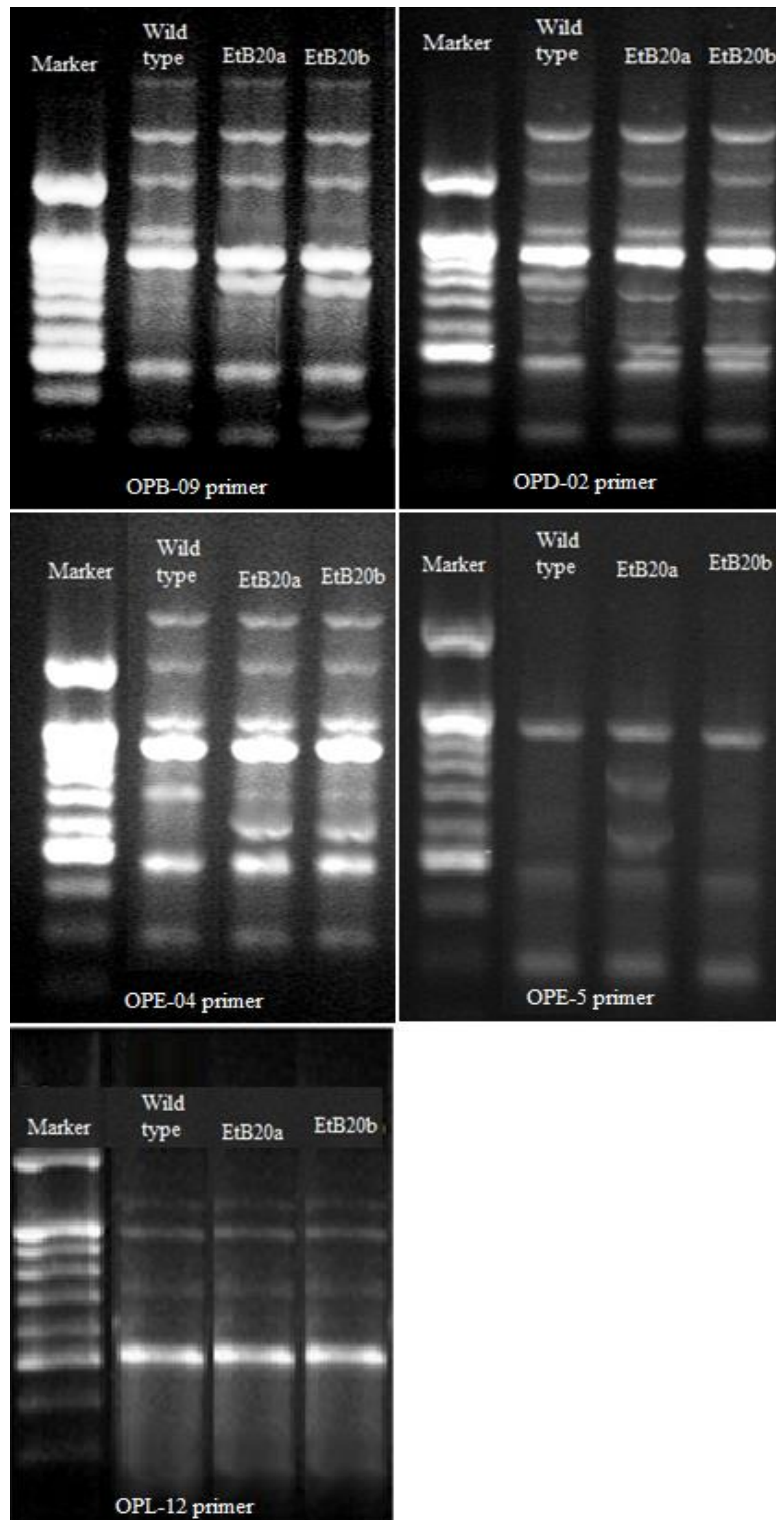


Fig. 4. Agarose gel electrophoresis of primers used for comparison of EtB20a and EtB20b mutants and wild-type of *S. cerevisiae*

While according to the OPD-02 primer, most of the bands were detected in the wild-type and mutants except band 819.334 base pair molecular weight was detected only in the wild-type. At the same time, a band with 550.900 base pair molecular weight was

detected only in the mutant strains. With using the OPE-04 primer, a band with 828.762 base pair molecular weight was found in the wild-type while a band with 622.609 base pair molecular weight was found in the mutants, the rest of the bands were detected in both wild-type and mutants. Three bands were detected in the mutants and wild-type according to bands of the OPE-5 primer, while EtB20a mutant was characterized by the presence of two bands with 469.506 and 330.852 base pair molecular weights. No difference between the bands was detected in wild-type and mutants with OPL-12 primer. Amplification with the primers indicated the presence of polymorphisms, allowing the presence of dissimilar profiles between the wild-type and mutants. Mutagenesis of *S. cerevisiae* by EtBr has been studied before (Pinto *et al*. 1975). As it was reported by Pfeiffer *et al*. (2010) and Sayas *et al*. (2015), EtBr stimulates the highest loss of mitochondrial DNA and can induce massive formation of petite (non-respiratory) mutants, as well as stimulates the decomposition of already existing mtDNA molecules. Thus, it is recommended that the RAPD technique is enough to differentiate between the wild-type and the mutant strains.

At a laboratory scale, the wild-type and mutant strains (EtB20a and EtB20b) of *S. cerevisiae* were tested for ethanol production (Table 3), and conditions were optimized. Mutant EtB20a produced 9.07% ethanol and mutant EtB20b produced 19.5%, while wild-type produced 18.01% with sugar utilization of 20.76, 40.06, and 41.24%, respectively. The results clearly demonstrated that ethanol production by mutant EtB20a and EtB20b increased with increasing sugar concentration up to 40%, unlike that of the wild-type. The authors' results were in agreement with Liu *et al.* (2011), who reported that ethanol production was remarkably enhanced through the chemical mutagenesis of *S. cerevisiae* in very high-gravity fermentation. Transcriptome studies of *S. cerevisiae* wild-type and its mutant showed that the ethanol tolerance is due to the highest levels of oxidative reactions in the mitochondria (Stanley *et al.* 2010b). The mutant strain EtB20b showed better results than those demonstrated by the wild-type at all sugar concentrations. The authors found that the mutant EtB20a did not exhibit an improved capacity for ethanol production, but rather was more sensitive to osmotic stress than the wild-type strain. The inhibitory effect was consequently due to high osmotic pressure that inhibited the growth of yeast cells combined with decreasing the overall ethanol production**.**

Table 3. Ethanol Production and Glucose Utilization by EtB Selected Mutants

Selected Mutant	Medium with Sugar (%)	Ethanol Production (%)	Glucose Utilization (%)
Wild-type	30	18.01h	41.24bc
	40	17.92h	42.98a
	50	17.05i	40.05c
EtB20a	30	9.07j	20.76d
	40	12.28k	20.79d
	50	9.78m	20.02e
EtB20b	30	19.50g	40.06c
	40	20.34f	41.75b
	50	20.22f	40.80bc

Means followed by the same letter are not significantly different

Optimization of ethanol production and glucose utilization (%) by mutant EtB20b at different temperatures, pH, incubation periods (h), and inoculum sizes were estimated using a high-gravity medium (Figs. 5 through 8). In the experiments conducted within this

study, a sharp increase in ethanol production by the mutant EtB20b was observed with increasing temperature up to 30 °C, and then it decreased with increasing temperature, although the ethanol production (%) was best at 40 °C (13.27%) compared to 20 °C (8.04%) (Fig. 5). Earlier studies (Anderson *et al.* 1986; Pereira *et al.* 2011) found that temperature tolerant yeast can still produce more than 6% ethanol within 24 h at 40 °C. In the current study, the EtB20b can be regarded as mildly thermo-tolerant. As previously reported in literature, ethanol production was reduced at high temperature due to transport system changes that can cause increasing toxic accumulation inside the cell (Lin *et al.* 2012). The obtained results indicated that mutant EtB20b was able to produce ethanol at high temperature. Morimura *et al.* (1997) reported that *S. cerevisiae* at 35 °C was able to ferment molasses containing sugar up to 20% (w/v); however, with a sugar concentration of 22% (w/v) the fermentation was inhibited. Higher temperature restricts the fermentation process because the majority of yeast strains do not tolerate temperatures beyond 40 °C. Through genome shuffling technique and mutagenesis, *S. cerevisiae* was genetically modified to become more resistant to elevated temperature and very high-gravity fermentations (Hou 2010; Pattanakittivorakul *et al.* 2019). Applying the same technique, Shi *et al*. (2009) reported the generated strain of *S. cerevisiae* fermented substrate with 20% (w/v) glucose at 45 °C. Mutant RPRT90 of *S. cerevisiae* exhibited the highest tolerance to ethanol up to 10% and good growth at high temperature (39 to 40 °C) (Kumari and Pramanik 2012). Ethanol production increased up to a pH of 6.0 and then decreased as the pH increased (Fig. 6). The highest three percentages of ethanol production, 19.9, 19.5, and 19.1% were obtained at pH 6, 5, and 7, respectively, a highly alkaline substrate with pH 8 and 9 was favorable for ethanol production than an acidic substrate (pH 3 and 4). Lin *et al.* (2012) reported that pH 4 to 5 was optimum for *S. cerevisiae* growth but for the ethanol production pH 6 was the best. According to Sivakumar *et al.* (2010), pH 4 was determined to be optimum for ethanol production by wild-type *S. cerevisiae*.

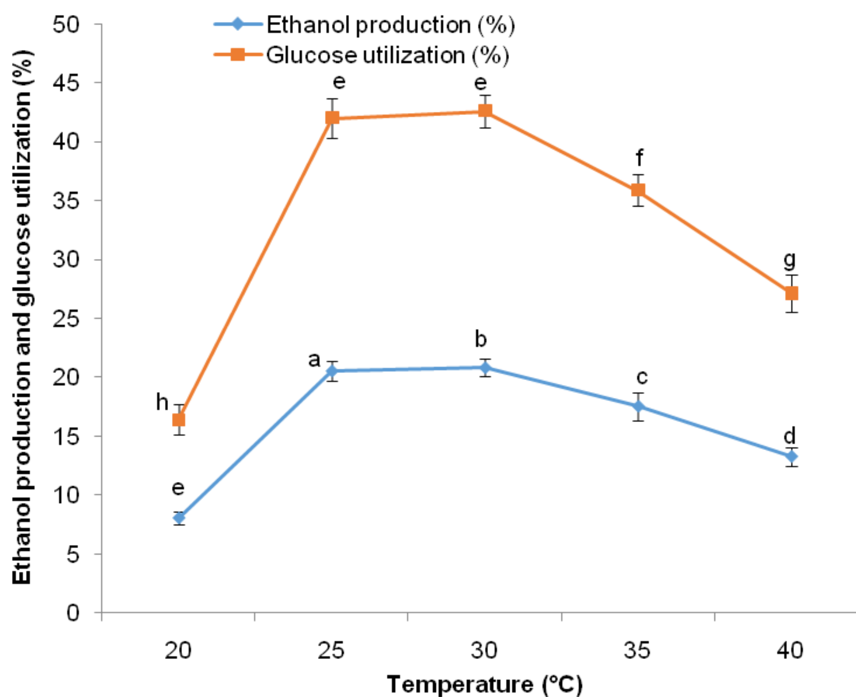


Fig. 5. Optimization of ethanol production and glucose utilization (%) by mutant EtB20b at different temperature (°C); means followed by the same letter are not significantly different in each series

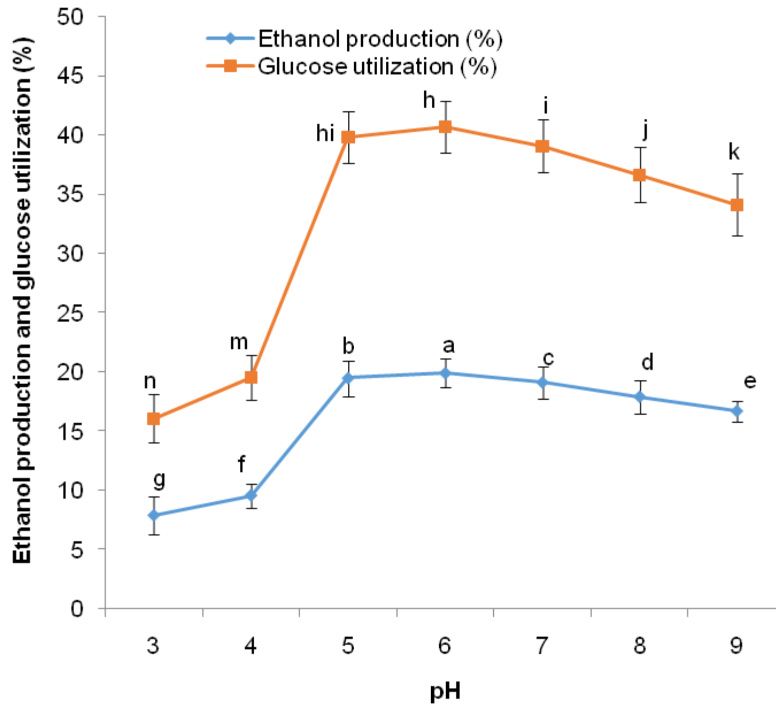


Fig. 6. Optimization of ethanol production and glucose utilization (%) by mutant EtB20b at different pH; means followed by the same letter are not significantly different in each series

As presented in Fig. 7, inoculum size (mL) was an efficient factor for ethanol production; however, the expected results were opposite of the obtained results, where the increasing inoculum size reduced the ethanol yield.

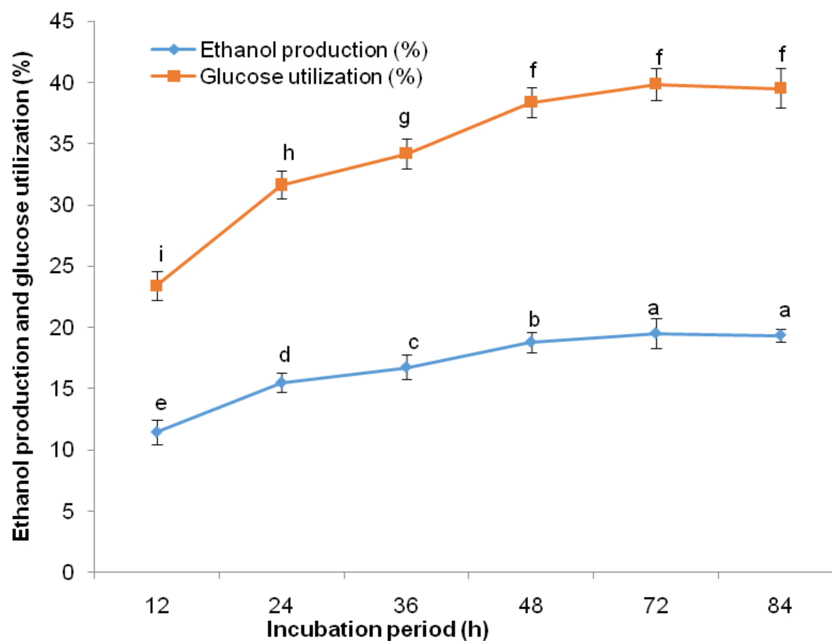


Fig. 7. Optimization of ethanol production and glucose utilization (%) by mutant EtB20b at different incubation period (h); means followed by the same letter are not significantly different in each series

Optimum inoculum size was 1 mL, followed by 1.5 and 0.5 mL. Ethanol concentration increased with increasing incubation period up to 72 h, then decreased at 84 h. Although the production at 84 h was not remarkable, it decreased compared with production at 27 h (Fig. 8). Therefore, the consumed glucose was not significantly different at 48, 72, and 84 h of incubation. The authors' results were in agreement with previous studies (Brooks 2008; Sivakumar *et al.* 2010). The decreasing ethanol concentration at the end of the incubation period may have been due to accumulation of toxins or increasing the ethanol production, which inhibited yeast growth and therefore the productivity of ethanol will be affected. Although it has been recorded in an earlier study (Ingledew 2009), ethanol inhibition could not affect the ethanol productivity even if a yeast growth inhibition occurred.

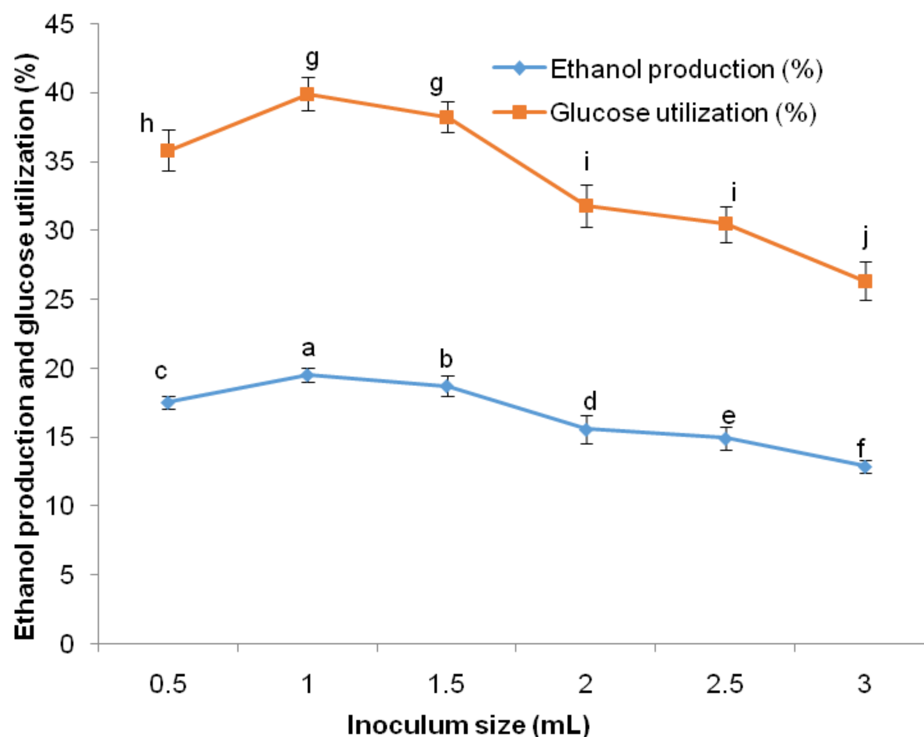


Fig. 8. Optimization of ethanol production and glucose utilization (%) by mutant EtB20b at different inoculum size (mL); means followed by the same letter are not significantly different in each series

CONCLUSIONS

1. *S. cerevisiae* EtB20b exhibited an increase of ethanol production, which supports the observation that mutagenesis is one of the promising techniques to improve ethanol productivity by *S. cerevisiae*, particularly under VHG fermentation.
2. *S. cerevisiae* EtB20a exhibited retardation to ethanol production in comparison to wild-type. This implies that the mutant(s) used for ethanol production improvement should be carefully chosen.

3. Ethanol production of EtB20b was improved through optimization of culture conditions. Under optimization and favorable conditions, the ethanol production was improved.
4. Finally, the impact of EtB is possibly related to the fact that the areas of genes responsible for osmotic stress resistance and fermentation of sugars by *S. cerevisiae* were affected.

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Article submitted: October 20, 2019; Peer review completed: December 31, 2019; Revised version received and accepted: January 3, 2020; Published: January 8, 2020.
DOI: 10.15376/biores.15.1.1354-1369